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Plant Cell Rep (2002) 20:1033–1038
DOI 10.1007/s00299-001-0431-y

GENETIC TRANSFORMATION AND HYBRIDIZATION

D.W. Darnowski · L.O. Vodkin

A soybean lectin-GFP fusion labels the vacuoles in developing *Arabidopsis thaliana* embryos

Received: 25 July 2001 / Revised: 3 December 2001 / Accepted: 3 December 2001 / Published online: 22 February 2002

Abstract We report the use of a derivative of the green fluorescent protein (GFP) to directly label the plant vacuole in live, unfixed tissues of stably transformed transgenic plants. We used the developmentally regulated soybean seed lectin promoter and the 32 amino acids of the soybean lectin amino terminal signal sequence to create an in-frame fusion polypeptide with GFP (pLGFP5). This construct was transferred into *Arabidopsis thaliana* by vacuum infiltration, and the transformed lines were characterized by DNA blotting and immunoblotting to detect the presence and expression of the GFP gene. GFP fluorescence was detected in the protein storage vacuoles of developing *Arabidopsis* embryos as imaged by fluorescence microscopy. Very little signal was detected in any other compartments including the cell wall. Thus, despite the absence of vacuolar sorting signals in GFP and other foreign proteins fused to the lectin sequence, the 32-amino-acid lectin signal sequence has general utility to direct foreign proteins to the protein storage vacuoles in seeds.

Keywords Localization in protein storage vacuoles · Soybean lectin signal sequence · Green fluorescent protein

Abbreviations *CaMV*: Cauliflower mosaic virus · *GFP*: Green fluorescent protein · *ER*: Endoplasmic reticulum · *β-GUS*: β-Glucuronidase · *PCR*: Polymerase chain reaction · *SDS-PAGE*: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis · *VSDs*: Vacuolar sorting determinants

Communicated by E.D. Earle

D.W. Darnowski · L.O. Vodkin (✉)
Department of Crop Sciences,
University of Illinois at Urbana-Champaign,
Urbana, IL 61801, USA
e-mail: l-vodkin@uiuc.edu
Tel.: +1-217-2446147, Fax: +1-217-3334582

Present address:
D.W. Darnowski, Department of Biology, Washington College,
300 Washington Ave., Chestertown, MD 21620, USA

Introduction

The plant vacuole serves as a storage site for many products, including the storage proteins of the seed that supply energy during germination of the seedling. This paper reports the use of a 32-amino-acid N-terminal signal sequence from the soybean seed lectin gene (Lindstrom et al. 1990; Vodkin et al. 1983) to label *Arabidopsis thaliana* storage vacuoles in developing seeds with the autofluorescent GFP protein. Instead of being exported by the default pathway, the GFP is found in the protein storage vacuoles of the *Arabidopsis* embryos.

GFP was first discovered in the autofluorescent jellyfish *Aequoria victoria* (Chalfie et al. 1994). It requires no externally applied substrate for fluorescence nor any chromophore besides a spontaneously cyclized trio of amino acids (Reid and Flynn 1997) normally found in the protein. These properties make GFP an ideal marker for in vivo studies of transgenic organisms.

In plants, GFP has previously been used to label a variety of subcellular compartments, including the cytoplasm (Haseloff et al. 1997; Pang et al. 1996), mitochondria (Gàlvez et al. 1998; Köhler et al. 1997b), chloroplasts (Köhler et al. 1997a), the phragmoplast (Gu and Verma 1997), the endoplasmic reticulum (Boevnik et al. 1996), and the vacuole of transformed protoplasts or tissue culture cell lines (Di Sansebastiano et al. 1998, 2001; Mitsuhashi et al. 2000). GFP has also been used to follow phenomena such as the spread of viruses through the plant body (Epel et al. 1996; Oparka et al. 1995). For most of these studies in plants, it has been necessary to use a mutant form of GFP that has amino acid substitutions enhancing fluorescence and maintaining correct RNA processing (Haseloff et al. 1997; Siemering et al. 1996; Zhang et al. 1998).

Seed lectins form one important category of proteins found in seed storage bodies derived from vacuoles, i.e. protein storage vacuoles. Soybean lectin is a 120,000-Da homotetramer composed of 30,000-Da polypeptide subunits that are glycosylated as they pass through the ER. A 32-amino-acid N-terminal sequence is cleaved from

the nascent lectin polypeptides and is consequently not found in the mature protein (Vodkin et al. 1983). Soybean lectin is localized in the protein bodies of soybean seed (Vodkin and Raikhel 1986).

We have constructed embryo-specific expression cassettes in which the 5′ and 3′ non-translated regions of the lectin gene can be utilized with or without the 32-amino-acid signal sequence (Cho et al. 1995) and have shown that the reporter enzyme β-glucuronidase appeared to be localized in the protein storage vacuoles of the cotyledons of tobacco seeds (*Nicotiana tabacum* L. Solanaceae) when it was fused to the 32-amino-acid lectin N-terminal signal sequence (Philip et al. 1998). More extensive results have been conducted on localization and processing of the bovine β-casein polypeptide fused to the 32-amino-acid N-terminal lectin sequence in transgenic soybean seeds (Philip et al. 2001). The 32-amino-acid lectin sequence is cleaved precisely from the casein polypeptide, and casein is localized in the protein storage vacuole of the developing soybean seeds.

In this report, GFP5 was fused to the lectin signal and used to transform *Arabidopsis thaliana*. These experiments demonstrate the action of this construct in live embryos rather than using fixed tissue and present the first localization of GFP within vacuoles of intact plant tissues as opposed to transformed protoplasts or cell lines. More importantly, they directly contrast the observed vacuolar localization of GFP5 when fused to the 32-amino-acid lectin sequence to that of the GFP5ER construct that contains HDEL, an ER retention signal. The default pathway for proteins with only a hydrophobic N-terminal signal sequence and no vacuolar sorting determinants or ER retention signals is generally considered to be export from the cell. However, we have now shown that the 32-amino-acid lectin signal sequence has a general utility to direct vacuolar localization in seeds as evidenced by using three very different foreign proteins (GFP, casein, and β-GUS) in three different species.

Materials and methods

Construction of pBINLGFP5

The plasmid pBIN m-gfp5-ER contains the modified GFP5 coding region with improved fluorescence and thermostability. This construct also contains an *Arabidopsis* chitinase signal sequence at the amino terminal end of the coding region as well as the four-amino-acid ER retention signal (HDEL) at the carboxy terminus that results in targeting of the protein to the lumen of the ER (Siemering et al. 1996; Zhang et al. 1998); expression of GFP is driven by the CaMV 35S promoter. DNA of the pBIN m-gfp5-ER plasmid was amplified by PCR using primers designed to omit both the chitinase signal sequence and the ER retention signal and to add *Not*I cloning sites to each end of the GFP coding region. The sequences of the forward and reverse primers used were the following, respectively: gfp5a – 5′ aaggaaaaaagcggccgccAGTAAAGGAGAAGAACTT 3′; gfp5b – 5′ ttcctttttgcggccgcTTATTTGTATAGTTCATC 3′. The region corresponding to the GFP5 coding region is in capital letters, and the *Not*I restriction site is underlined.

Using the *Not*I sites, we inserted this GFP5 construct into the *Not*I sites of the lectin expression cassette pGL-3 (Cho et al. 1995)

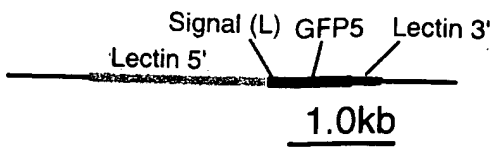

Fig. 1 Diagramatic representation of the lectin-GFP expression region contained in pLGFP5 and pBINLGFP5. The GFP coding region was PCR-amplified using *Not*I adapters and fused in-frame to the pGL expression cassette (Cho et al. 1995) that contains the 32-amino-acid lectin signal sequence (*Signal L*) that is flanked by the 1.7-kb lectin gene promoter and 5′ region (*Lectin 5′*) and 326-bp 3′ region (*Lectin 3′*)

to produce pGLGFP5 (Fig. 1). The orientation and single copy number of the GFP insert was determined by automated sequencing. A 3.0-kb *Eco*RI/*Sal*I fragment of pGLGFP5 that contains the lectin-GFP region was cloned into the pBIN19 T-DNA multicloning site to make pBINLGFP5. The insert orientation was confirmed by sequencing, and copy number was confirmed by restriction enzyme digestion.

Transformation of *Arabidopsis thaliana* and control plant lines

Wild-type plants of the *Arabidopsis thaliana* Columbia ecotype were grown to the flowering stage and vacuum-infiltrated with *Agrobacterium tumefaciens* strain GV3101 carrying the pBINLGFP5 plasmid (Bechtold et al. 1993; Bent and Clough 1998). Mature seeds from infiltrated plants were screened on plates of YT medium containing 50 mg l^{-1} kanamycin.

Based on our observations of *Arabidopsis thaliana* siliques, silique development was divided into three stages – immature (silique elongating and swelling), intermediate (full length and girth silique), and mature (tan siliques). This division was used in following the expression of GFP through development.

A. thaliana Columbia ecotype plants transformed with the pBIN m-gfp5-ER construct (a gift from Steven Clough and Andrew Bent) and expressing GFP5ER in their ER were grown under the same regime as the pLGFP5 transformants. Material from these plants was used for positive controls, while material from *A. thaliana* Columbia ecotype non-transformed plants was used for negative controls.

DNA blotting

Genomic DNA was extracted from leaves or seedlings and Southern blotting performed according to Cho et al. (1995). DNA was digested with *Eco*RI and *Sal*I to release a 3.0-kb lectin-GFP fragment or with *Kpn*I or *Cla*I. The probe was random primer-labeled (GibcoBRL, Grand Island, N.Y) using the gel-purified 3.0-kb *Eco*RI/*Sal*I fragment of pBINLGFP5 as a template.

Protein immunoblotting

Proteins from mature seeds, leaves, and various silique developmental stages from pBINLGFP5-transfomed *A. thaliana* plants were extracted by grinding in phosphate-buffered saline (10 m*M* Tris HCl pH 7.0, 123 m*M* NaCl, 0.1% Triton X-100). Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, Calif.). For protein immunoblots, 5 μg or 10 μg of total protein from each sample, the same amount being used from each line for a given organ, was separated on 5% stacking, 10% resolving SDS-PAGE gels according to Sambrook et al. (1989). Immunoblotting was performed according to Darnowski et al. (1996) except that we used 3E6 anti-denatured autofluorescent (GFP) protein monoclonal antibody (Quantum Biotechnologies, Montreal, Quebec) as a primary antibody and alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, St. Louis, Mo.) as the second-

ary antibody, followed by developing with NBT/BCIP (Philip et al. 1998).

Fluorescence microscopy

Wet-mounts of live, manually dissected *A. thaliana* parts were examined by conventional means using an Olympus BH-2 fluorescence microscope (Olympus America, Melville, N.Y.) equipped with PlanApo phase contrast optics and fluorescence filters (either UG-1 excitation and a 455-nm barrier for DAPI staining or a 490-nm excitation and 515-nm barrier for GFP fluorescence or acridine orange staining). Micrographs were recorded on Kodak 160 T film since transmitted light and fluorescence micrographs were interleaved in rolls of film. Developed transparencies were scanned using ADOBE PHOTOSHOP 5.0 driving an AGFA Duoscan (AGFA Division, Bayer, Wilmington, Mass.) using a gamma value of 1.8 at a minimum of 1,000 dpi. The only digital adjustments made to images were to trim photographs for placement in Fig. 4, to adjust the levels to maximize the range, and to adjust the brightness and contrast.

Results

Selection and confirmation of transgenic *Arabidopsis* lines

As described in the Materials and methods, we modified the GFP found in the pBIN m-gfp5-ER plasmid to remove the amino terminal chitinase signal sequence and the carboxy-terminal ER retention HDEL sequence. The amplified GFP5 coding region was inserted into a cassette containing the lectin 5′ region, the 32-amino-acid lectin signal sequence, and the lectin 3′ terminator region (Fig. 1). In vitro translation of the lectin-GFP fusion plasmid with and without microsomal membranes showed that it was translatable and that the signal sequenced appeared to be cleaved from the lectin-GFP fusion as it was from a similar lectin-casein fusion construct (Philip et al. 2001). This insert was placed into a binary vector to form pBINLGFP5, which was used to transform *Arabidopsis* by vacuum infiltration.

Twelve transgenic T2 families were selected from the seed produced by the plants subjected to vacuum infiltration. T1 is the designation for the infiltrated plant according to normal nomenclature used with this technique. Based on initial examinations and antibiotic resistance segregation, several lines were selected for further analysis. Most of the analyses, including DNA blotting and protein immunoblotting, were performed on lines from families 1, 4, and 11 in the T3 or T4 generations. Family 1 appears to have inserts at multiple loci from the DNA blotting, and antibiotic resistance segregation results (Fig. 2; left). Some lines within family 1 gave particularly strong fluorescence, including lines 1-1 and 1-6. Families 4 and 11 each seem to have insert(s) at a single locus based on segregation data (not shown) and DNA blotting results (Fig. 2).

All of the transformed lines used for microscopic analysis showed a single band for GFP on a protein immunoblot at apparent molecular weight of approximately

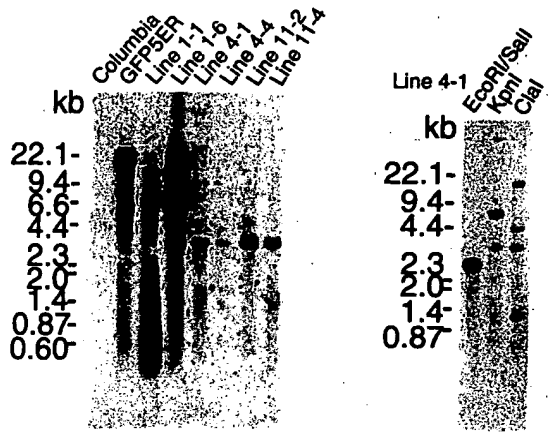

Fig. 2 DNA blots of genomic DNA from pBINLGFP5-transformed *Arabidopsis thaliana* genomic DNA. *Left panel* Genomic DNA (8 μg of DNA per lane) from various lines transformed with pBINLGFP5 were double-digested with *Eco*RI and *Sal*I to release the 3.0-kb *Eco*RI/*Sal*I fragment containing the lectin-GFP gene and probed with a random primer-labeled probe made against the corresponding fragment. GFP5ER served as a positive control, and the Columbia wild type served as a negative control. *Right panel* Genomic DNA from line 4-1, which showed single locus inheritance of kanamycin resistance, was digested with *Eco*RI/*Sal*I, *Kpn*I, or *Cla*I and probed with the lectin-GFP gene as above

25–28 kDa (Fig. 3, right). All of the pBINLGFP5 (lectin-GFP) lines that were tested by protein immunoblotting expressed GFP protein in developing seeds, with protein expression being maximal at the middle and late stages of silique development (Fig. 3, middle). GFP protein was not detected in the leaves of any plants transformed with the lectin-GFP construct but was detected in the leaves of the GFP5ER plants in which the constitutive 35S promoter drives expression (Fig. 3, left).

Detection of GFP fluorescence in vacuoles of developing *Arabidopsis* seeds

GFP fluorescence is not visible in immature embryos but can be seen in those from an intermediate stage of development. In mature seed, strong autofluorescence of the seed coat masks GFP fluorescence. Because of this strong autofluorescence and because of complications in working with dissected mature seed, the intermediate stage of development was chosen for microscopic studies.

The panels of Fig. 4 contrast the localization of GFP as directed by the GFP5ER construct as opposed to the LGFP5 construct in which GFP expression is driven by the soybean lectin promoter and signal sequence. Figure 4a shows GFP fluorescence in whole seedlings of transgenic lines that express the GFP5ER protein under control of the 35S promoter. In this line, the GFP has a chitinase signal sequence to direct it to the ER and also an HDEL sequence at the carboxy terminal end to specifically retain the protein in the lumen of the ER. Figure 4f shows the subcellular localization of the GFP fluorescence in the epidermal cells of developing embry-

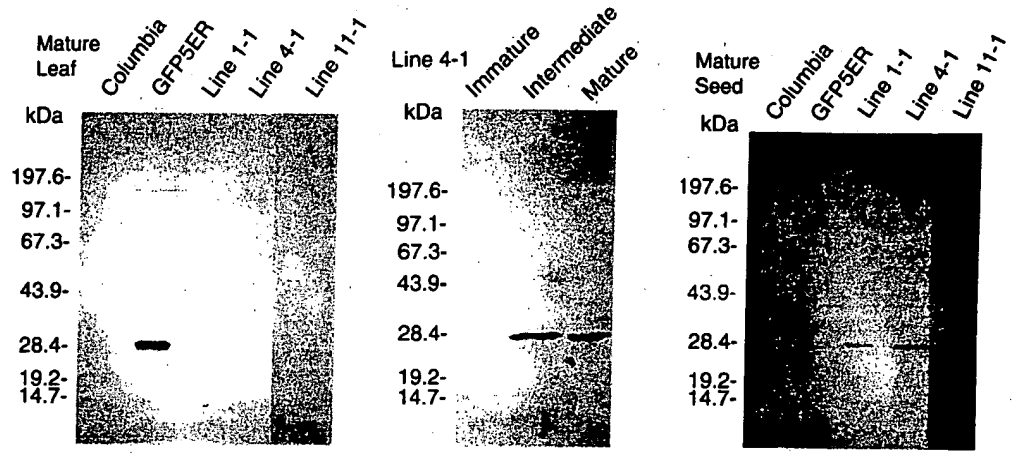

Fig. 3 Protein immunoblots of pBINLGFP5-transformed *A. thaliana* extracts. *Left panel* Total proteins from mature leaves of the Columbia wild type (negative control), from GFP5ER-expressing plants with the 35S-driven GFP construct, and from various LGFP5-expressing lines containing the lectin-GFP fusion were immunoblotted and probed with antibodies to GFP. *Middle panel* Protein from immature, intermediate, and mature stages of silique development for line 4-1 was immunoblotted to detect GFP expression. *Right panel* Protein from mature seeds of lines 1-1, 4-1, and 11-1, line GRP5ER, and Columbia were probed with antibodies to GFP

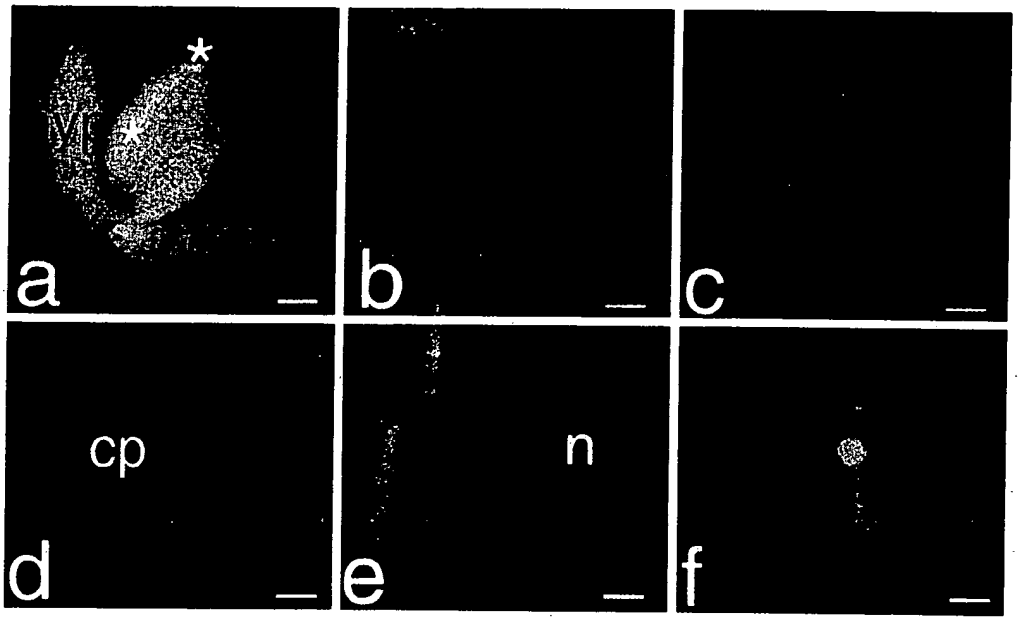

Fig. 4a–f Fluorescence detection of GFP expression in the vacuoles of lectin-GFP (LGFP5) transformed *A. thaliana* seed during intermediate stages of embryo development: panel **c** as compared to GFP5ER-transformed seeds and various controls. **a** Overview of GFP5 fluorescence in a seedling transformed with the GFP5ER construct that contains an endoplasmic reticulum (ER) retention signal at the carboxy-terminal end of the GFP protein. GFP expression is patchy, with an especially bright stripe running between the *asterisks*. *cot* Cotyledon *hyp* hypocotyl. **b** Vacuoles of cotyledonary epidermal cells of non-transformed Columbia embryos stained with acridine orange. *V* Greenish-yellow vacuoles, one per cell in most cases. **c** Strong GFP fluorescence in the vacuoles (*V*) in the epidermal cotyledonary cells of lectin-GFP line 1-1 transformed seed with the LGFP5 construct that contains the lectin signal sequence. **d** Fluorescence image of non-transformed control, Columbia wild-type embryonic cotyledon. Autofluorescence of the chloroplasts (*cp*) is evident. **e** DAPI-stained non-transformed Columbia wild-type embryonic cotyledon. Nuclei (*n*) are blue, and some few small organellar genomes are also stained. **f** GFP fluorescence in cotyledon cells transformed with the GFP5ER construct that contains an ER retention signal at the carboxy-terminal end of the GFP protein. *Bars*: **a** 100 μm; **b–f** 10 μm

os transformed with the GFP5ER construct. The ER localization pattern that was typically observed was that of small granules typical of ER cisternae and an occasional large, round structure that could be aggregated ER cisternae or aggregated fusion protein.

In contrast, Fig. 4c shows GFP fluorescence detected in epidermal cells of developing embryos of a line transformed with the LFGFP5, the lectin-GFP fusion. GFP-specific fluorescence is seen in large bodies that are similar in size to the primary vacuole of the cell. The vacuoles